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Short-term responses of denitrification to chlorothalonil in riparian sediments: Process, mechanism and implication

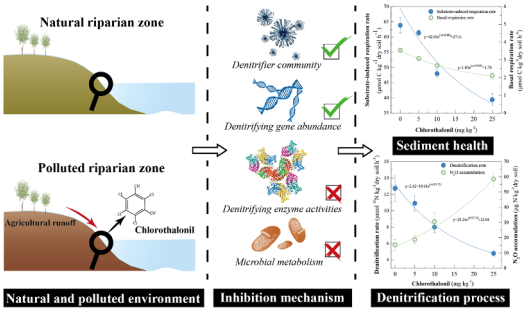
Yi Chen[a,b], Xiaoxuan Su[a,b], Yiyu Wang[a,b], Shuyuan Zhao[a,b], Qiang He[a,b,*]

[a] Key Laboratory of the Three Gorges Reservoir Region's Eco-Environment, Ministry of Education, Chongqing University, Chongqing 400045, China
[b] College of Urban Construction & Environmental Engineering, Chongqing University, Chongqing 400045, China

HIGHLIGHTS

- Short-term effects of pesticide on riparian denitrification were investigated for the first time.
- CTN declined sediment denitrification rate but increased N_2O accumulation after 48 h.
- CTN remarkably inhibited microbial metabolism during denitrification.
- NAR, NIR and NOS activities were significantly declined by CTN.
- Riparian CTN potentially impacts nearby aquatic environments and climate changes.

GRAPHICAL ABSTRACT



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ABSTRACT

Pesticide residues in riparian zones have attracted much attention in recent decades. Their accumulations potentially deteriorate microbial activity and disturb nitrogen cycle in riparian sediments. In this study, the short-term effects of chlorothalonil (CTN, a common pesticide) on microbial denitrification were explored in riparian sediments at three levels (5, 10 and 25 mg kg^{-1}). No significant differences were observed between control and 5 mg kg^{-1} treatment; however, CTN in 10 and 25 mg kg^{-1} treatments deteriorated sediment health condition and microbial activity. High concentrations of CTN also significantly decreased denitrification rates (^{15}N pairing method) by 37–62%, but increased N_2O emission in riparian sediments by 100–285%. Our data further revealed that CTN inhibited key enzyme activities responsible for microbial metabolism, and declined electron donor (NADH) and energy source (ATP) levels during denitrification. Key denitrifying enzyme activities were also suppressed by CTN, which explained the declined denitrification process and the elevated N_2O emission. Additionally, high-throughput sequencing and quantitative-PCR analysis showed that CTN didn't remarkably change microbial community structures and denitrifying gene abundances after short-term exposure. Overall, this study highlights that riparian pesticides could impact nitrogen cycle of the interface between terrestrial and aquatic ecosystems, potentially accelerating water pollution and global climate change.

1. Introduction

Pesticides have been widely used in modern agricultural production to protect crops from diseases and pests [1,2]. Chlorothalonil (2,4,5,6-tetrachlorobenzene-1,3-dicarbonitrile, CTN) is the second popular fungicide in the world, and its annual application has been nearly 5000 t in U.S. and more than 8000 t in China [3]. Less than 0.3% of CTN could reach the targets after application, and 99.7% are released into soil or other

* Corresponding author at: 174 Shazhengjie Street, Shapingba District, China.
E-mail address: heqiang@cqu.edu.cn (Q. He).

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ecosystems [4]. Along with agricultural runoff, CTN residues are discharged into nearby aquatic environment via riparian zones [5–7]. Thus, CTN residues have been frequently detected in soils, waters and sediments. Because of its toxicity, CTN has received much attention in recent decades [3,8]. Riparian CTN residues probably influence sediment health, potentially disturbing ecological functions and element biogeochemical cycles.

Riparian zone is a unique ecosystem, connecting the terrestrial and aquatic environments [9]. The interface between the two adjacent ecosystems is often the hotspot for element cycles [10]. Thus, riparian zone has long been viewed as biogeochemical hot zone of nitrogen transformations, and is important for nitrogen balance in the whole biosphere [9–11]. Fluctuating hydrological regime and intensive substance exchange between land and water provide ideal conditions for a series of biotic and abiotic processes, which are related to nitrogen cycle: mineralization, nitrification, and denitrification [12]. CTN accumulations potentially disturb microbial activity and nitrogen transformation processes in riparian sediments. Previous studies have indicated that CTN could change soil community structure, deteriorate microbial activity, and influence extracellular enzyme activity [8,13]. Such adverse effects further endanger soil nitrogen transformations. For example, Lang and Cai reported that CTN applications at 20 times and 40 times field rates significantly declined soil nitrification rates in different soil types [14]. Zhang also documented that fungicide CTN remarkably downregulated *amo*A, *amo*B and *nif*H gene abundances in soil, which are related to microbial nitrification and nitrogen fixation processes [3]. Although the toxicity of CTN to soil microbial community and nitrification has been reported, fewer studies were found about the effects of pesticide on denitrification process in riparian sediments.

Microbial denitrification consists of four step bioreductions, which are catalyzed by NO_3^- reductase (NAR), NO_2^- reductase (NIR), NO reductase (NOR) and N_2O reductase (NOS), respectively [15,16]. This process can reduce reactive nitrogen in riparian sediments, but also lead to the potent greenhouse gas N_2O emission [11,17]. It has been documented that denitrifiers require to consume electron and energy to finish denitrification reactions [15,18]. Nicotinamide adenine dinucleotide (NADH) and adenosine triphosphate (ATP) are direct electron donor and energy source for denitrification, which are generated through microbial metabolism [19]. Therefore, microbial metabolism plays an important role in denitrification process. The produced NADH could be directly catalyzed by NADH dehydrogenase (complex I) in electron transport system to produce electrons [20]. In this system, the electron flows are then transferred via quinone pool and electron transport protein [15,21]. After transport, these electrons are finally utilized by the four key denitrifying enzymes. Previous studies have indicated that denitrifying enzyme activities had great influences on denitrification process [18,21]. Pesticide residues probably deteriorate microbial metabolic activity and denitrifying enzyme activity, potentially influencing sediment denitrification and N_2O emission. However, the impacts of riparian pesticides on denitrification and N_2O emission at microbial metabolism level have never been documented yet.

With these considerations, we collected riparian sediments from the Three Gorges Reservoir to explore the effects of CTN on denitrification and N_2O emission at microbial metabolism level. The specific objectives of the study are to: (i) explore CTN accumulation and dissipation behaviors in riparian sediments; (ii) investigate the short-term effects of CTN on sediment health and denitrification process; (iii) reveal impact mechanism of CTN on sediment denitrification. We hypothesize that CTN may significantly deteriorate riparian denitrification during the short-term exposure experiment by directly inhibiting microbial metabolic activity and denitrifying enzyme activity in denitrifiers.

2. Materials and methods

2.1. Riparian sediment sampling and characterization

Riparian sediments were collected in June 2017 from the riparian zones of the Three Gorges Reservoir (Chongqing area), China (Supplementary Materials, Fig. S1). Three Gorges Reservoir, the largest artificial reservoir in the world, has 306 km^2 of riparian zones [22]. In this reservoir, 38% of the areas are agricultural land use. Agricultural non-point source pollutants can be discharged into the reservoir via riparian zones. Hence, it provides a good opportunity to study the effects of CTN on denitrification. Four typical sampling sites: Guangyang (29°33′ N 106°41′ E), Zhongxian (30°26′ N 108°11′ E), Wushan (31°16′ N 109°47′ E) and Kaixian (31°11′ N 108°26′ E) were selected. Sampling points and their location characteristics were depicted in Fig. S1. Immediately after collecting, sediment cores (15 kg) were brought back to laboratory in coolers and analyzed for physicochemical characteristics. Sediment characteristics are pH: 7.66 ~ 8.07, soil moisture: 80 ~ 85%, TN: 0.75 ~ 1.28 mg kg^{-1}, NO_3^--N: 6.37 ~ 21.07 mg kg^{-1}, NO_2^--N: 0.11 ~ 1.52 mg kg^{-1}, NH_4^+-N: 2.33 ~ 4.66 mg kg^{-1}, SOM:7.67 ~ 13.87 mg kg^{-1}. Background CTN concentrations vary from 0 to 4.03 mg kg^{-1}. Sediments from the four sampling sites were then mixed together and stored at 4 °C until experiments.

2.2. Experimental preparation and set-up

CTN standardized substance (CAS NO. 1897-45-6) was purchased from J&K Scientific, China. CTN for experiment was purchased from Aladdin Scientific, China. To test the short-term effects of CTN on denitrification in riparian sediments, a series of exposure experiments were conducted. In this study, three levels (5, 10 and 25 mg kg^{-1}) of CTN were selected, and sediments without CTN addition were used as control. Specifically, for more accurate and convenient in the following experiments, 5 mg kg^{-1} is selected to represent the basal CTN concentration. 10 and 25 mg kg^{-1} represent the cumulative concentrations of CTN in the future. Furthermore, high concentrations of pollutants could help us to bound the threshold levels between better and worse case scenarios.

In detail, 50 g of riparian sediments were transferred to 150-mL serum bottles. CTN dissolved in Milli-Q water was sprayed to sediments to reach the concentrations of 5, 10, 25 mg kg^{-1}, respectively (T5, T10 and T25 treatments). Equal volume of distilled water was added to control group. Each treatment was set in quintuplicates. Initial carbon and nitrogen sources were 5 mg g^{-1} glucose and 120 mg kg^{-1} NO_3^--N. The exposure experiment was incubated at 28 °C in the anaerobic glove box for 48 h with 80% water content. During this period, NO_3^--N, NO_2^--N and NH_4^+-N concentrations in sediments were measured at 0, 4, 8, 12, 20, 28, 36, and 48 h. In this study, we took destructive sampling strategy and there were a total of 160 bottles for the incubation (4 treatments × 8 sampling times × 5 quintuplicates). At the end of the experiment, CTN concentration, N_2O accumulation, sediment basal and substrate-induced respiration rate, denitrification rate (based on ^{15}N isotope pairing technique), enzyme activity, electron transport system activity (ETSA), glucose utilization, NADH level, ATP level, pyruvate level, denitrifying gene abundance, and microbial community structure were detected.

2.3. Chemical analysis

After 48 h, CTN residues in sediments were extracted as described by Chaves [23] and Hladik [24]. Briefly, 5 g of samples were extracted with acetone–hexane solution (1:1, volume ratio) by the ultrasonic method for thrice. Solutions were then concentrated by rotary evaporation for 2.5 h. After filtering with anhydrous sodium sulfate column (Na_2SO_4), the solution was filtered with 0.45-μm filters. CTN was measured by using GCMS-TQ8040 (Shimadzu, Japan) with DB-5MS column (30 m length × 0.25 mm i.d. × 25 μm phase thickness). The recovery rate is 92.3–106.8%. Detection limit of CTN is 5 μg kg^{-1}, and quantitation limit is 10 μg kg^{-1}. Detail procedures were in Supplementary Materials. Glucose concentration was measured by using the anthranone method [18]. Anthranone-H_2SO_4 was added to 3 g of sediment samples. Then the mixture was boiled at 100 °C for 10 min.

The supernatants were collected at 5000 rpm and measured at 620 nm using an ultraviolet spectrophotometer (DR5000, HACH, USA). Water and sediment physicochemical characteristics (NO_3^-, NO_2^-, NH_4^+, pH, moisture, SOM, and TN) were determined following our previous study [2,25].

2.4. Molecular biological analysis

After the 48 h experiment, key enzyme activities (glucokinase (GK), glyceraldehyde-3-hosphate dehydrogenase (GAPDH), NO_3^- reductase (NAR), NO_2^- reductase (NIR), NO reductase (NOR), and N_2O reductase (NOS)) involved in microbial metabolism and denitrification process were determined. Briefly, 5 g of sediments were washed with 100 mM phosphate-buffered saline (PBS) (pH = 7.8) for three times, and re-suspended at 4 °C. Sonication method was used to disrupt the suspensions at 4 °C for 5 min. Then, the supernatants were collected by centrifugation at 16000 rpm at 4 °C for 10 min, and were immediately used to measure enzyme activities. Protein contents in sediment samples were determined using Total Protein Assay Kit (Nanjing Jiancheng Institute of Biological Engineering, China) with bovine serum albumin as a standard.

GK activity was determined by measuring NADPH formation at 340 nm according to previous study [18]. The assay mixture (2 mL) contained 0.1 M Tris-HCl buffer (pH = 7.5), 0.5 mM NADP, 10 mM $MgCl_2$, 1 mM ATP, 10 mM glucose, and 2 U glucose-6-phosphate dehydrogenase. 1 mL of enzyme extract was injected to start the reaction. GAPDH activity was determined by measuring NADH formation at 340 nm [19,26]. The assay mixture (2 mL) contained 0.01 M Tris-HCl buffer (pH = 8.5), 0.02 M sodium arsenate, 2 mM DL-glyceraldehyde-3-phosphate, 2 mM NAD, and 0.5 mM dithiothreitol. Then, 1 mL of enzyme extract was injected to start the reaction, and the absorbance was recorded every 30 s for 5 min. GK and GAPDH activity was presented as nm mg^{-1} protein min^{-1}.

NAR, NIR, NOR and NOS activities were measured according to a previous study [18]. Assay mixture (3 mL) contained 10 mM PBS buffer (pH = 7.8), 10 mM methyl viologen, 5 mM $Na_2S_2O_4$ and 1 mM denitrifying electron acceptor (NO_3^-, NO_2^-, NO or N_2O). Next, enzyme extract (1 mL) was added to start the reaction. All these denitrifying enzyme assays were conducted at 25 °C under anaerobic condition. After 30 min, the increased or decreased NO_2^- concentration was determined at 540 nm to calculate NAR and NIR activities. The produced or reduced N_2O concentration was detected by the microsensor (MMM-Meter, Unisense, Denmark) to calculate NOR and NOS activities. Denitrifying enzyme activity was presented as μmol N g^{-1} dry soil h^{-1}.

ETSA was measured by the method for reducing 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride to formazan according to previous studies [21]. Absorbance of the orange-like formazan was immediately recorded at 490 nm against a solvent blank. Detail procedures were provided in Supplementary Materials. NADH content was measured using enzymatic cycling assay as described in the literature [19,27]. The absorbance was recorded every 30 s at 570 nm for 10 min. Detail procedures were provided in Supplementary Materials. ATP levels in sediments were measured following our previous study [2]. Briefly, 20 mL of the trichloroacetic acid-HPO_4^{2-} were added to samples to extract ATP, then immediate sonification for 2 min. ATP was measured using ATP Assay Kit (Nanjing Jiancheng Institute of Biological Engineering, China). Pyruvate was extracted using the method identical to intracellular enzyme extraction as mentioned above. Pyruvate concentration was measured with pyruvate assay kit (NO. A081) provided by Nanjing Jiancheng Institute of Biological Engineering, China.

After 48 h, 10 g of sediments were collected from control and T25 treatment to detect denitrifying gene abundances and community structures. Details for DNA extraction were given in previous publications [28,29]. Denitrifying functional genes *nar*G, *nir*K/S, *nor*B and *nos*Z were quantified by real-time quantitative PCR (ABI 7500). Information of the primers for the denitrifying functional genes was listed in our previous study [2]. Microbial community structures in sediments were detected by using Illumina MiSeq sequencing of the 16S rRNA gene. The details of pyrosequencing and analysis have been presented in previous studies [30,31].

2.5. Respiration rate and denitrification rate

After 48 h, basal respiration rate and substrate-induced respiration rate in riparian sediments were measured according to the literature [1]. At the end of the assay, the produced CO_2 was collected and analyzed by gas chromatography (GC-2010Plus, Shimadzu, Japan). Denitrification rate was measured by using ^{15}N isotope pairing technique according to previous study [32]. At the end of the assay, $^{29}N_2$ and $^{30}N_2$ gases were taken and analyzed by isotope ratio mass spectrometry (MAT-253, Thermo, USA). More details were listed in Supplementary Materials.

2.6. Statistical analysis

In this study, all assays were conducted in quintuplicates and the results were normally distributed. Statistical differences were determined by one-way ANOVA with the least significant difference post-hoc test (SPSS 22.0). Data were considered significant when p was < 0.05.

3. Results

3.1. CTN residues and dissipation in riparian sediments

In this study, basal CTN contents in riparian sediments were investigated. Background concentrations of CTN varied from 0 mg kg^{-1} in Zhongxian to 4.03 mg kg^{-1} in Guangyang. Fig. S2 showed the CTN residues in each treatment after 48 h exposure experiment. In detail, CTN residues were 3.98 ± 0.93, 9.07 ± 0.67, 23.84 ± 1.21 mg kg^{-1} in T5, T10 and T25 treatments, respectively. CTN dissipation rates were 0.51 mg kg^{-1} d^{-1} in T5, 0.49 mg kg^{-1} d^{-1} in T10, and 0.58 mg kg^{-1} d^{-1} in T25 (one-way ANOVA, $p > 0.05$), indicating no significant differences among CTN-treated sediments.

3.2. Impacts of CTN on sediment respiration activity and microbial community structure

After 48 h, basal and substrate-induced respiration rates were measured to investigate the short-term effects of CTN on sediment health and microbial activity. Fig. 1A showed that CTN remarkably declined basal respiration rates from 3.53 μmol C kg^{-1} dry soil h^{-1} in control to 2.10 μmol C kg^{-1} dry soil h^{-1} in T25 treatment (one-way ANOVA, $p < 0.05$). For substrate-induced respiration rate, no significant differences were found between control and T5 (one-way ANOVA, $p > 0.05$) (Fig. 1B). However, CTN in T10 and T25 treatments significantly decreased substrate-induced respiration rates (one-way ANOVA, $p < 0.05$) (Fig. 1B). For microbial community structure in riparian sediments, no obvious differences were detected between control and CTN-treated sediments, regardless of phylum and genus levels (Fig. S3).

3.3. Impacts of CTN on denitrification process in riparian sediments

Fig. S4 showed the variations of NO_3^-–N, NO_2^-–N, and NH_4^+–N concentrations in control and CTN-treated sediments during the 48 h experiment. At the end of the experiment, no statistical differences for NO_3^--N concentrations were found between control (1.21 mg kg^{-1}) and T5 (2.93 mg kg^{-1}) (One-way ANOVA, $p > 0.05$) (Fig. S4A). However, NO_3^--N accumulations were remarkably increased in T10 and T25 treatments (one-way ANOVA, $p < 0.05$). High concentrations of CTN

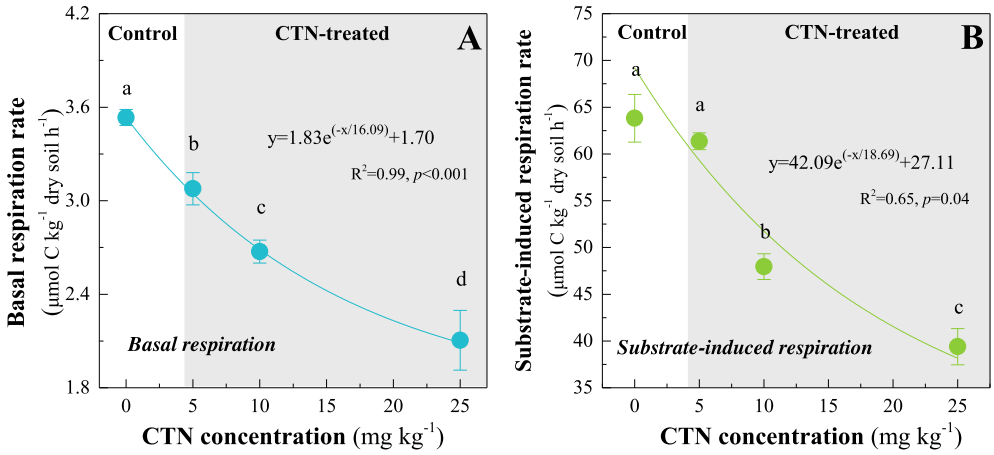

Fig. 1. Effects of CTN on basal respiration rate (A) and substrate-induced respiration rate (B) in riparian sediments. Error bars denote standard deviations of five independent tests (n = 5). Different letters indicate the significant differences among treatments ($p < 0.05$, one-way ANOVA followed by LSD test).

also inhibited NO_2^-–N reduction (Fig. S4B). In detail, NO_2^--N concentrations were 5.40 and 12.82 mg kg^{-1} in T10 and T25 treatments, respectively, significantly higher than 0.79 and 1.22 mg kg^{-1} in control and T5 treatments (One-way ANOVA, $p < 0.05$). NH_4^+–N concentrations remained relative constant during the exposure experiment (Fig. S4B), regardless of control and CTN-treated sediments.

In T10 and T25 treatments, CTN remarkably declined denitrification rates by 37.2% and 62.4%, respectively (one-way ANOVA, $p < 0.05$) (Fig. 2A). At the end of the experiment, however, high concentrations of CTN (10 and 25 mg kg^{-1}) promoted N_2O accumulations from 15.23 μg kg^{-1} dry soil in control to 58.49 μg kg^{-1} dry soil in T25 treatment (one-way ANOVA, $p < 0.05$) (Fig. 2B). No significant differences were measured between control and T5 treatment, regardless of denitrification rate and N_2O accumulation (one-way ANOVA, $p > 0.05$).

For denitrifying enzyme activities, NAR, NIR and NOS activities were inhibited when high concentrations of CTN accumulated in riparian sediments (Fig. 3A, B and D). NAR, NIR and NOS activities were 0.143, 0.657 and 5.416 μmol N g^{-1} dry soil h^{-1} in control, higher than 0.067, 0.167 and 2.075 μmol N g^{-1} dry soil h^{-1} in T25 treatment (one-way ANOVA, $p < 0.05$). However, CTN had no detectable impact on NOR activity (one-way ANOVA, $p > 0.05$) (Fig. 3C). Additionally, relative abundances of *nar*G, *nir*S, *nir*K, *nor*B and *nos*Z were also quantified in this study. Results showed that CTN did not significantly affect the five denitrifying functional gene abundances (one-way ANOVA, $p > 0.05$) (Fig. 3E–H).

3.4. Impacts of CTN on microbial carbon source metabolism in riparian sediments

In the present study, the stress responses of microbial carbon source metabolism (glycolysis and TCA cycle) to CTN were investigated to reveal the inhibition mechanism. Schematic diagram of metabolism pathways was depicted in Fig. 4, and the corresponding data were provided in Fig. 5. After 48 h, no measurable differences in glucose utilization and GK activity were detected between control and CTN-treated sediments (one-way ANOVA, $p > 0.05$) (Fig. 5A and B). However, high concentrations of CTN remarkably inhibited GAPDH activity (one-way ANOVA, $p < 0.05$) (Fig. 5C), which catalyzes the key step of glyceraldehyde-3-phosphate to 3-phosphogylcerate in glycolysis. In detail, GAPDH activities were declined from 0.163 nm mg^{-1} protein min^{-1} in control to 0.077 nm mg^{-1} protein min^{-1} in T25 treatment. Pyruvate levels in microbes were also decreased by CTN in T10 and T25 treatments, approximately 35% and 60% lower than that of control (one-way ANOVA, $p < 0.05$) (Fig. 5D). Electron donor (NADH) and energy source (ATP) contents were measured in this study as well (Fig. 5E and F). NADH and ATP in control were 12.14 μmol g^{-1} dry soil and 5.28 μmol kg^{-1} dry soil, respectively, significantly higher than 4.22 μmol g^{-1} dry soil and 1.03 μmol kg^{-1} dry soil in T25 treatment (one-way ANOVA, $p < 0.05$). Additionally, CTN in T10 and T25 treatments remarkably inhibited electron transport system activity (ETSA) by 31.7% and 43.5% (one-way ANOVA, $p < 0.05$) (Fig. 6). During the experiment, we found that T5 treatment

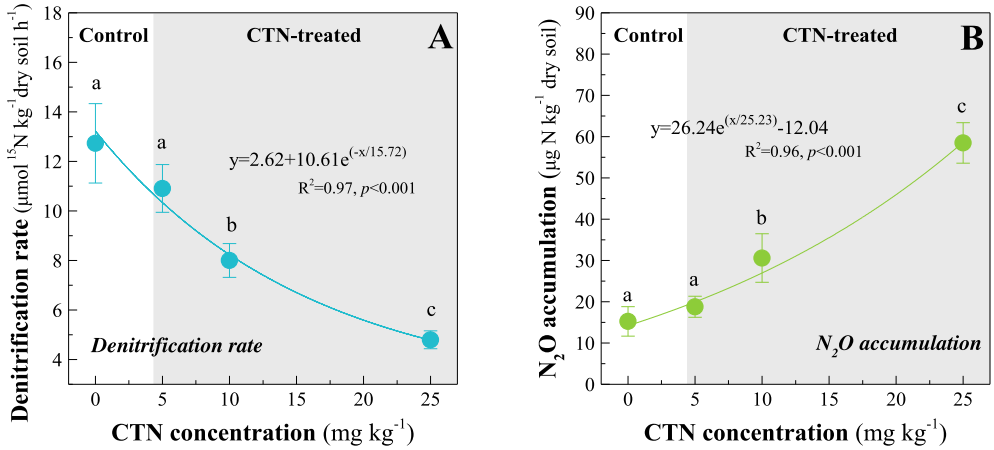

Fig. 2. Effects of CTN on denitrification rate (A) and N_2O accumulation (B) in riparian sediments. Error bars denote standard deviations of five independent tests (n = 5). Different letters indicate the significant differences among treatments ($p < 0.05$, one-way ANOVA followed by LSD test).

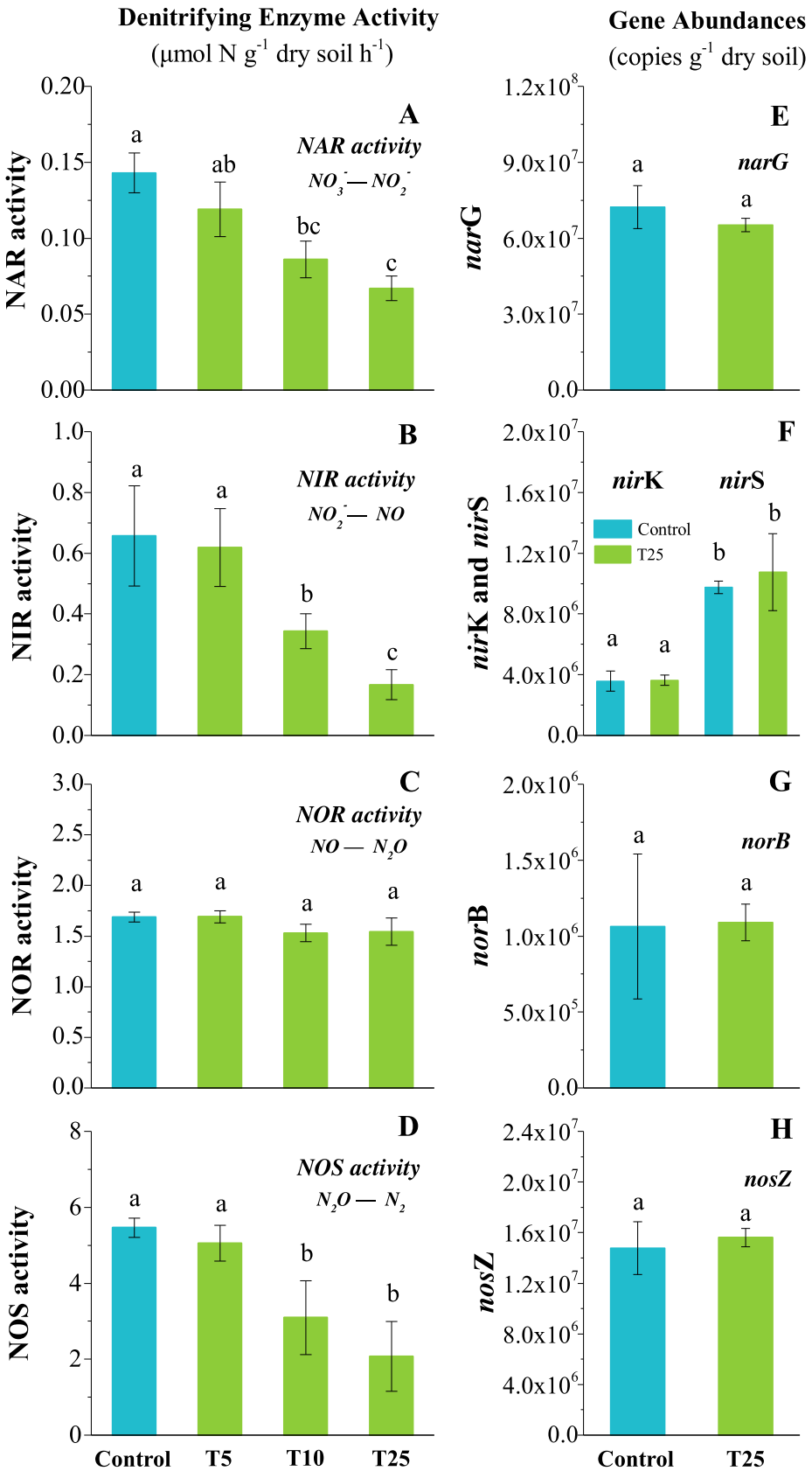

Fig. 3. Effects of CTN on denitrifying enzyme activities (A–D) and denitrifying gene abundances (E–H) in riparian sediments. Error bars denote standard deviations of five independent tests (n = 5). Different letters indicate the significant differences among treatments ($p < 0.05$, one-way ANOVA followed by LSD test).

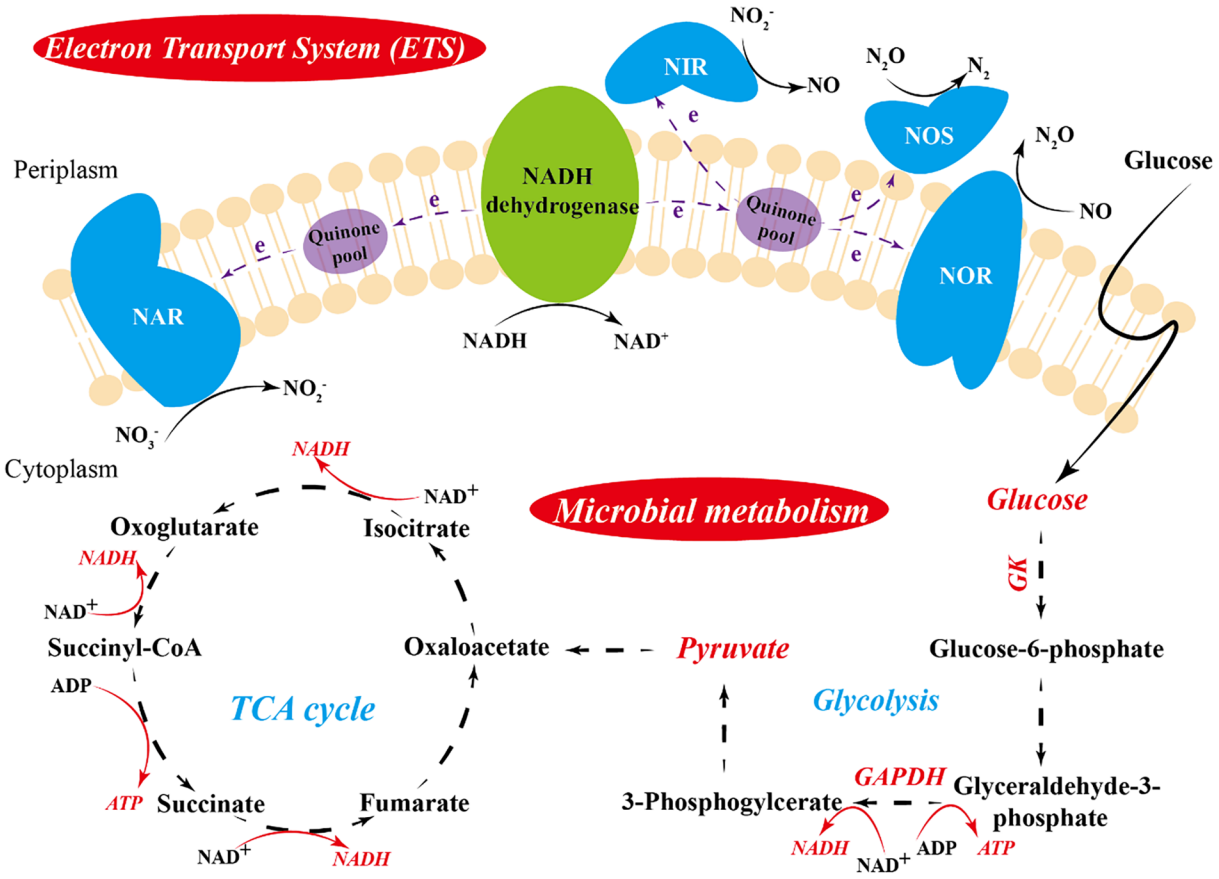

Fig. 4. Schematic diagram of canonical microbial organic matters metabolism and electron transport system during denitrification.

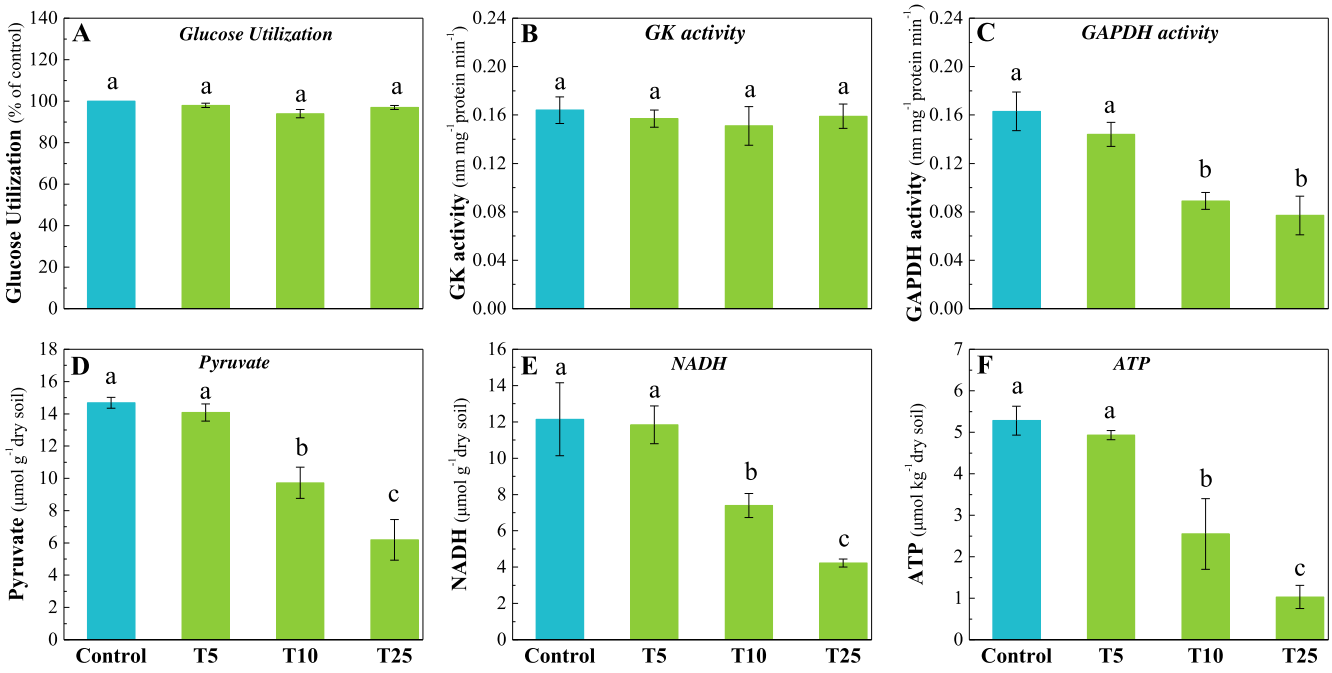

Fig. 5. Effects of CTN on glucose utilization (A), GK activity (B), GAPDH activity (C), pyruvate level (D), NADH content (E) and ATP level (F) in riparian sediments. Error bars denote standard deviations of five independent tests (n = 5). Different letters indicate the significant differences among treatments ($p < 0.05$, one-way ANOVA followed by LSD test).

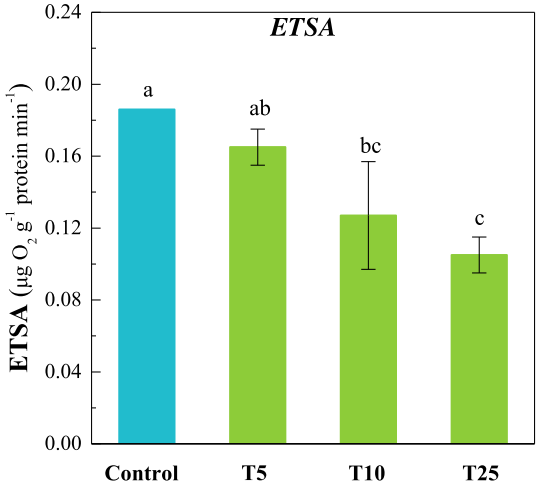

Fig. 6. Effects of CTN on electron transport system activity (ETSA). Error bars denote standard deviations of five independent tests (n = 5). Different letters indicate the significant differences among treatments ($p < 0.05$, one-way ANOVA followed by LSD test).

had no obvious inhibitions on microbial metabolism compared to control.

3.5. Relationships between denitrification, and electron production, transport and consumption

Denitrification process had significant correlations with the capacity of electron production, transport, and consumption in riparian sediments (Fig. 7). In detail, denitrification rate had the positive relationships with NADH (electron donor) and GAPDH activity (catalyzing to produce NADH in glycolysis), with R^2 ranging from 0.847 to 0.891 (Fig. 7A). Furthermore, Fig. 7B showed that denitrification rate also had the positive relationship with ETSA (electron transport) ($R^2 = 0.599$, $p = 0.03$). For electron consumption, denitrification rate was positively correlated with NAR, NIR and NOS activities, with R^2 ranging from 0.382 to 0.829 (Fig. 7C and D). However, no significant relationship between denitrification rate and NOR activity was observed ($p = 0.07$) (Fig. 7D).

4. Discussion

Riparian zones are ecologically important environments and can often receive non-point source pollutions which are enriched with large amounts of nitrate [12,33]. The increased nitrate loads in this region pose a great threat on aquatic ecosystem when water table fluctuates. In riparian zones, the fluctuation of water table provides an ideal redox condition for denitrification to remove nitrate, but this process is easily disrupted by human activities [12,32]. Previously, most research on denitrification in riparian sediments focused on the effects of natural factors (e.g., water table fluctuating, seasonal variations, and vegetation species) [11,12,34], but frequently neglected the roles of human activities, such as pesticide accumulations. This study, for the first time, has indicated that pesticide CTN accumulation could cause the negative impacts on microbial denitrification in riparian sediments.

CTN residues from agricultural runoff have the strong potential to adsorb onto sediment surfaces, and such adsorption by sediment particles may be the major pathway of CTN dissipation under the shot-term exposure [8,35]. Our study indicated that high concentrations of CTN (T10 and T25 treatments) obviously accumulated in sediments, and thus declined sediment health condition (Fig. 1). It has been also documented that soil microbial activity could be remarkably inhibited when CTN residues was over 10 mg kg^{-1} [3], which further confirmed our results. Besides CTN, previous studies have also reported that pesticides glyphosate [36], tebuconazol [1], hexaconazole [37] and difenoconazole [38] could deteriorate soil health condition, inhibit microbial activity and decrease microbial biomass. High concentrations of pesticides deteriorated microbial activity in soils or sediments probably because they could increase environmental stress and decrease the

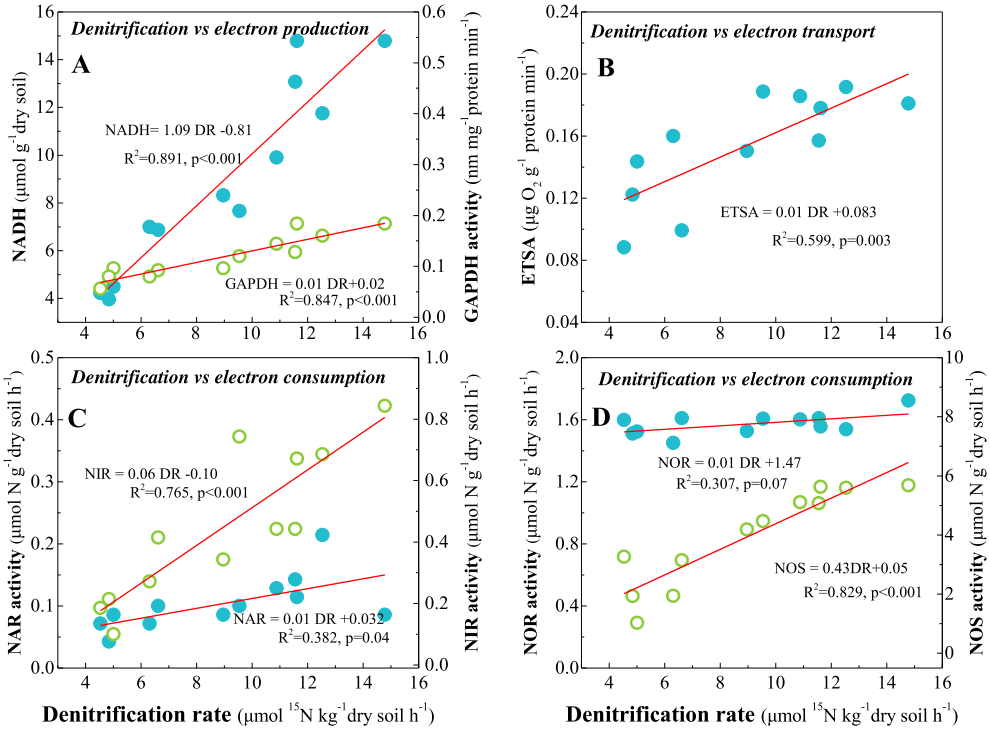

Fig. 7. Relationships between denitrification rate with electron production (A, NADH and GAPDH activity), electron transport (B, ETSA), and electron consumption (C and D, denitrifying enzyme) in riparian sediments.

energy required to relieve the environmental stress and survive [37].

Microorganisms in sediments are commonly regarded as the main driver for nitrogen cycle [38]. Such adverse impacts on sediment microbes caused by CTN potentially disturb denitrification process in riparian sediments. Our study showed that CTN significantly inhibited denitrification process but enhanced N_2O emission, similar with previous studies conducted for other contaminants. For example, Hou et al. reported that over 50 ng L^{-1} of antibiotic sulfamethazine remarkably inhibited denitrification rate of estuarine and coastal sediments, and stimulated N_2O release in a dose-response pattern [32]. Also, Shan et al demonstrated that antibiotic tetracycline significantly altered soil nitrate reduction process and increased N_2O emission by 33.1–83.0% [39].

Microbial denitrification consists of four step bioreductions, which are commonly driven by electrons [15,18,21]. In riparian sediments, denitrifiers need to degrade extracellular carbon sources to provide electrons for the complete reduction from nitrate to N_2. Glucokinase (GK), a vital kinase, is responsible for catalyzing the first step in carbon source metabolism: glucose to glucose-6-phosphate (Fig. 4) [18,19]. GK activity could thus directly impact carbon source utilization during denitrification. In this study, CTN did not influence GK activity (Fig. 5B), which might be the important reason for the no inhibition on glucose utilization (Fig. 5A). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is another important enzyme for catalyzing the critical step of glyceraldehyde-3-phosphate to 3-phosphogylcerate during carbon source metabolism [19]. This step is the only pathway to generate NADH (electron donor for denitrification) in glycolysis [20]. GAPDH activity is thus closely related to electron production. It has been reported that GAPDH activity could be disturbed by oxidative stress, carbon source, heavy metal, pesticide, and nanoparticle, which thus inhibits microbial metabolic activity [40,41]. Our study has also showed that CTN in T10 and T25 treatments significantly inhibited GAPDH activity (Fig. 5C), potentially impacting the capacity of electron production in denitrifiers. Significant positive correlations between denitrification rate and GAPDH activity under CTN accumulation further confirmed this result (Fig. 7A). Furthermore, pyruvate is a critically important metabolite in microbial metabolism connecting glycolysis and TCA cycle (Fig. 4). Previous studies have indicated that pyruvate synthesis in microorganisms is directly related to the production of NADH in TCA cycle, and influences the complete carbon source metabolism [42]. In this study, CTN significantly declined pyruvate contents (Fig. 5D), probably deteriorating TCA cycle in microbes and affecting NADH production. NADH is the important electron donor during denitrification process, which can be produced through glycolysis and TCA cycle as mentioned above [15,27]. The generated NADH can be delivered to electron transport system and catalyzed by NADH dehydrogenase to produce electrons for denitrification (Fig. 4) [15]. NADH concentrations were remarkably decreased by high concentrations of CTN (Fig. 5E). This implied that CTN could decline electron production capacity of denitrifiers in sediments, and so inhibits denitrification process.

In electron transport system, these produced electrons are indirectly transferred to four denitrifying enzymes via quinone pools (Fig. 4). Our study showed that sediment denitrification rate had the positive relationship with ETSA (Fig. 7B), indicating that electron transport system in denitrifiers had a great influence on denitrification process. A pure culture study also demonstrated that ETSA could determine the denitrification performance of *Paracoccus denitrificans* [21]. As seen from Fig. 6, ETSA in control was 0.186 μg O_2 g^{-1} protein min^{-1}, lower than 0.59–3.28 μg O_2 g^{-1} protein min^{-1} conducted in freshwater sediments [43]. Such divergence exists probably due to the differences in riparian ecosystem and aquatic ecosystem. However, ETSA was significantly declined after high concentrations of CTN accumulations (Fig. 6), which resulted in a lower electron transport efficiency and thus deteriorated sediment denitrification.

After transport, electrons can be finally utilized by four denitrifying enzymes: NAR (NO_3^- → NO–2), NIR (NO_2^- → NO), NOR (NO → N_2O) and NOS (N_2O → N_2). Fig. 7C and D also demonstrated that sediment denitrification was positively correlated with NAR, NIR and NOS activities. In the present study, CTN significantly inhibited NAR, NIR and NOS activities (Fig. 3), thus impacting the efficiency of electron consumption. The declined NIR and NOS activities could be the direct reason for NO_2^- and N_2O accumulations during the experiment. Generally, denitrifying enzymes NAR, NIR, NOR and NOS are mainly encoded by *nar*G, *nir*S/K, *nor*B and *nos*Z [27]. In this study, high-throughput sequencing and quantitative-PCR showed that CTN did not significantly change denitrifier communities and the denitrifying functional gene abundances (Fig. 3 and S3). In contrast, some previous studies have indicated that microbial structure and diversity could be changed after CTN accumulation [8,13]. The opposing observations are mainly caused by the test periods (short- or long-term exposure). In this study, sediment microbial communities could not rapidly respond to CTN threat during the short-term accumulation (48 h), and thus microbial structures and denitrifying gene abundances were not obviously changed. In previous work, however, microbial community structures were detected at 60th or 90th day after CTN applications. Hence, microbial community and diversity were changed. Overall, such responses confirmed our hypothesis that CTN inhibited nitrate reduction and promoted N_2O emission by directly disturbing microbial metabolism and key enzyme activities rather than altering denitrifier communities and gene abundances in riparian sediments.

Riparian zones are the hotspots for nitrogen biogeochemical cycle, and are critical for nitrogen balance in the biosphere [9,10]. Pesticide accumulations in this zone could negatively impact on land-water ecosystem health, impairing nitrogen cycle. Our results showed that CTN residues in riparian sediments resulted in NO_3^--N and N_2O accumulations, which is involved in two important environmental issues: water eutrophication and global warming. NO_3^--N accumulation could be enhanced by approximately 15 fold if CTN residues reach 25 mg kg^{-1}, potentially contributing to water pollution of nearby aquatic environment. Additionally, potent greenhouse gas N_2O emission from riparian sediments could be promoted by 4 fold after 25 mg kg^{-1} of CTN accumulation, which probably contributes to global warming and depletes atmospheric ozone. Overall. our study reveals the acute responses of denitrification and N_2O emission to pesticide in riparian sediments, and has an important implication for pesticide control in riparian zones.

5. Conclusion

This study explores the short-term impacts of pesticide on denitrification and N_2O emission in riparian sediments at microbial metabolism level. During the experiment, no significant differences were observed between control and T5 treatment. However, CTN in the high concentration treatments (10 and 25 mg kg^{-1}) significantly inhibited NO_3^--N reduction, and promoted N_2O emission. CTN inhibited sediment denitrification process by deteriorating microbial metabolism and key enzyme activities in microbes, rather than directly affecting microbial community and gene abundance. Furthermore, significant relationships between sediment denitrification rate and electron production, transport and consumption were observed. Our study highlights that pesticide accumulation in riparian sediments could pose a potential threat to nearby aquatic ecosystem and global climate change. Future studies should pay more attention to the responses of aquatic environment and global warming to pesticide accumulation in the transitional zone between terrestrial and aquatic ecosystems.

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Appendix A. Supplementary data

Supplementary data (Detailed methods and figures) to this article can be found online at https://doi.org/10.1016/j.cej.2018.10.148.

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